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Biological

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Force-induced Adrb2 in Periodontal Ligament Cells Promotes Tooth Movement

APPENDIX

MATERIALS & METHODS

Measurement of Tooth Movement Distance

At the termination of the experimental periods, the animals were sacrificed by pentobarbital sodium overdose. The maxillae were removed en bloc, fixed in 4% paraformaldehyde, and scanned by high-resolution MicroCT (Inveon MMCT, Siemens, USA). The orthodontic tooth movement (OTM) distance was measured between 2 easily located points—the midpoint of the distal-marginal ridge of the first molar and the midpoint of the mesio-marginal ridge of the second molar—from the occlusal view of the 3-dimensional reconstructed image (Appendix Fig. 1C). Measurement of tooth movement distance was independently conducted by 2 trained researchers who were blinded to the group design. The average of 2 measurements was calculated as the tooth movement distance.

Cell Culture of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells were separated from the buffy coats of healthy human donors by density gradient centrifugation using Ficoll-Hypaque (TBD Science, Tianjin, China). $CD11b^+$ peripheral blood mononuclear cells were sorted with CD11b microbeads (5130328012, MACS, USA) and allowed to adhere for 24 hr.

Histology and Immunohistochemistry

The fixed maxillae were demineralized in 15% EDTA and embedded in paraffin. Blocks were sagitally sectioned (4.5 μm) and stained with hematoxylin and eosin for routine histopathologic evaluation. For immunohistochemistry, serial transverse sections (4.5 μm) were made and sequenced between tooth cervix and cementum ($n = 5$). Antibody against Adrb2 (1:100, sc-9042, Santa Cruz Biotechnology) was used, and staining was conducted as previously described (Wu *et al.*, 2010).

Western Blot Analysis

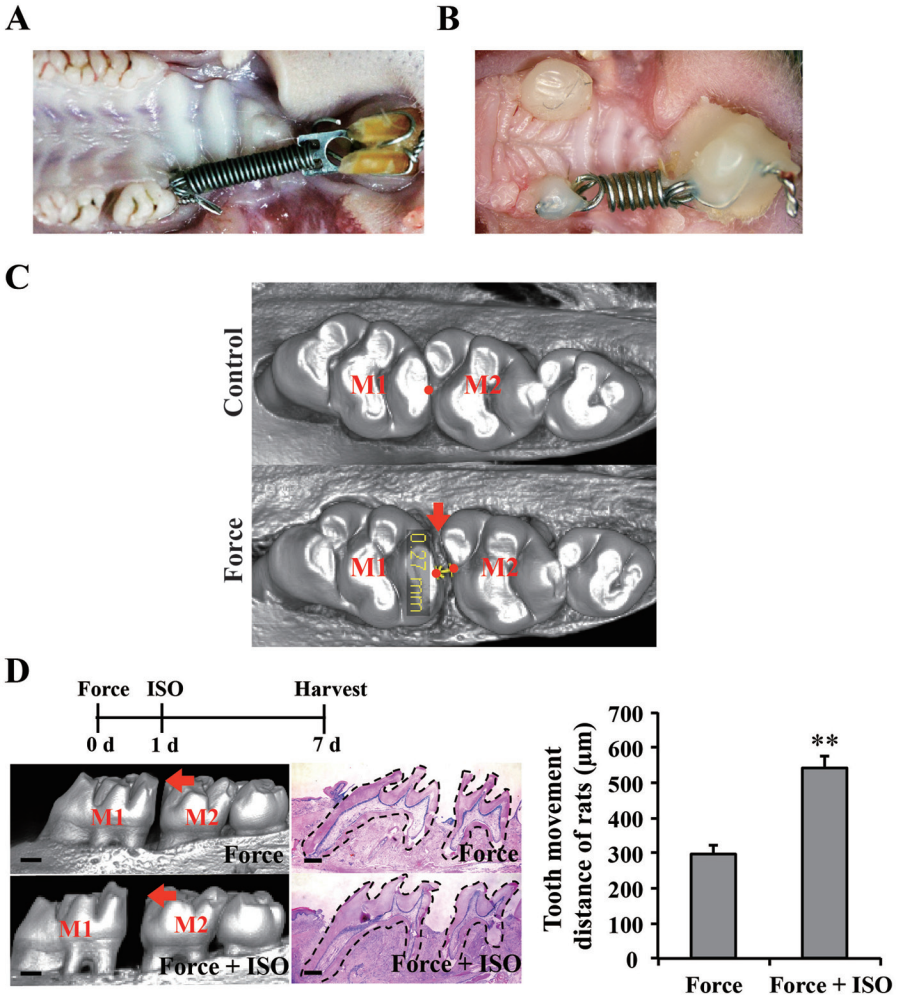
Western blot was performed as previously described (Wu *et al.*, 2010). Briefly, 25 μg of total protein was separated by 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane. After blocking in 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 for 1 hr at room temperature, the membranes were incubated with Adrb2 antibody (1:1,000), RANKL antibody (1:1,000, ab45039, Abcam), β-actin antibody (1:1000, a5441, Sigma), and α-tubulin (1:1,000, sc-398103, Santa Cruz Biotechnology) overnight at 4°C. The blots were developed using a horseradish peroxidase–conjugated secondary antibody and enhanced by chemiluminescence detection. Each experiment was repeated at least 3 times with comparable results. The fluorescence density was measured with ImageJ 1.37 software (Wayne Rasband).

Quantitative Real-time Polymerase Chain Reaction

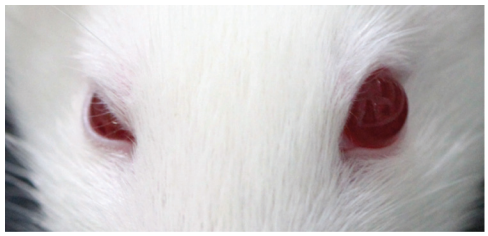
Total RNA was extracted with TRIzol Reagent (Invitrogen) in accordance with the manufacturer's instructions. Reverse transcription and real-time polymerase chain reaction was performed as previously described (Wu *et al.*, 2010). The primers used were as follows: human RANKL sense/antisense, 5′-AGAGCGCAGATGGATCCTAA-3′/5′-TTCCTTTTGCAC AGCTCCTT-3′; human OPG sense/antisense, 5′-GGAACCCCA GAGCGAAATACA-3′/5′-CCTGAAGAATGCCTCCTCAC A-3′; human GAPDH sense/antisense, 5′-ATGGGGAAGGTGA AGGTCG-3′/ 5′-ATGGGGAAGGTGAAGGTCG-3′; and human Adrb2 sense/antisense, 5′-CGCTACTTTGC CATTAC-3′/5′-CATAGGCTTGGTTCGT-3′. Primer efficiencies were confirmed by sequencing. Each experiment was repeated at least 3 times with comparable results.

Analysis of Intracellular Ca^{2+} Concentration in Response to Mechanical Stimulation

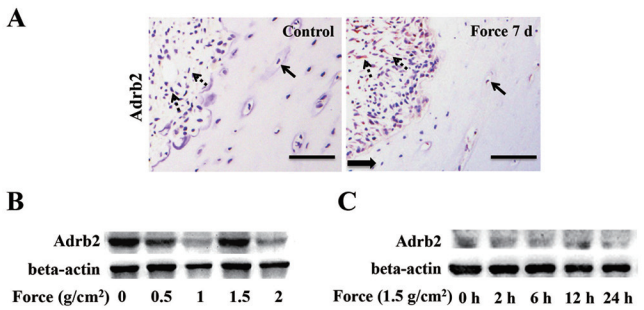
Periodontal ligament cells on a glass-covered dish were preincubated with 10 μM of Fluo3-AM (73881, Sigma) for 30 min at 37°C according to the manufacturer's protocol. Nuclei were counterstained with 10 μg/mL Hoechst 33258 (H6024, Sigma). The dish was set on the microscope, and periodontal ligament cells were subjected to 1.5 g/cm^2 of compressive force for 2 min in a Ca^{2+}-free or 10 mM Ca^{2+} medium. Confocal microscopy images were acquired using Zeiss laser scanning microscope (LSM 510). Fluorescence density was measured using Image-Pro Plus 6.0 software (Media Cybernetics).



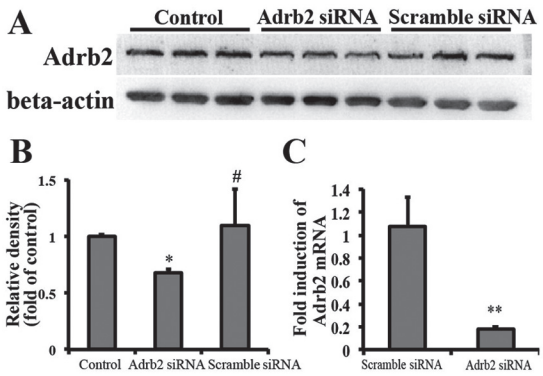
Appendix Figure 1. Orthodontic tooth movement (OTM) model in rats and mice. (**A**) Schematic of OTM model in rats. (**B**) Schematic of OTM model in mice. (**C**) Measurement of OTM distance. Red dots indicate the points that we located. Arrows indicate OTM distance. (**D**) Analysis of the OTM distance by micro–computed tomography scanning and hematoxylin and eosin staining. ISO injection increased OTM distance after 7 d of force application in rats (n = 10). ISO, isoproterenol. Hematoxylin and eosin staining shows the integrity of the periodontal tissue around the 2 molars after tooth movement.



Appendix Figure 2. Effectiveness of superior cervical ganglionectomy. The right eye shows ptosis of the upper eyelid after the right superior cervical ganglion was removed.



Appendix Figure 3. Force-induced increase of Adrb2 expression was not found in orofacial bone marrow–derived cells (OMCs). (**A**) Immunohistochemistry of β-2 adrenergic receptor (Adrb2). Orthodontic force increased the number of Adrb2-postive periodontal ligament cells in rats. But there was few increase of Adrb2 expression in OMCs. Bold arrow indicates the direction of tooth movement. Dotted arrows indicate periodontal ligament cells. Solid arrows indicate OMCs. The bar indicates 50 μm. (**B**, **C**) Western blot of Adrb2. Adrb2 expression in OMCs was not significantly upregulated with increasing force-treated intensity or time. Beta-actin served as internal control for equal loading.



Appendix Figure 4. Effectiveness of β-2 adrenergic receptor (Adrb2) siRNA transfection. (**A**) Western blot of Adrb2 in periodontal ligament cells. (**B**) Relative density of Adrb2 protein expression. Adrb2 siRNA transfection downregulated Adrb2 expression compared with that in the control and the Scramble siRNA transfection groups. *$p < .05$ *vs.* the control group. #$p < .05$ *vs.* Adrb2 siRNA transfection group. The data are representative of 3 independent experiments. (**C**) Real-time polymerase chain reaction data of Adrb2 in periodontal ligament cells. Adrb2 siRNA transfection downregulated Adrb2 mRNA expression in the Adrb2 siRNA transfection group. **$p < .001$.

APPENDIX REFERENCE

Wu YW, Bi YP, Kou XX, Xu W, Ma LQ, Wang KW *et al.* (2010). 17-Beta-estradiol enhanced allodynia of inflammatory temporomandibular joint through upregulation of hippocampal TRPV1 in ovariectomized rats. *J Neurosci* 30:8710-8719.